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NODULE ROOT and *COCHLEATA* Maintain Nodule Development and Are Legume Orthologs of *Arabidopsis BLADE-ON-PETIOLE* Genes[W][OA]

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During their symbiotic interaction with rhizobia, legume plants develop symbiosis-specific organs on their roots, called nodules, that house nitrogen-fixing bacteria. The molecular mechanisms governing the identity and maintenance of these organs are unknown. Using *Medicago truncatula nodule root* (*noot*) mutants and pea (*Pisum sativum*) *cochleata* (*coch*) mutants, which are characterized by the abnormal development of roots from the nodule, we identified the *NOOT* and *COCH* genes as being necessary for the robust maintenance of nodule identity throughout the nodule developmental program. *NOOT* and *COCH* are *Arabidopsis thaliana BLADE-ON-PETIOLE* orthologs, and we have shown that their functions in leaf and flower development are conserved in *M. truncatula* and pea. The identification of these two genes defines a clade in the BTB/POZ-ankyrin domain proteins that shares conserved functions in eudicot organ development and suggests that *NOOT* and *COCH* were recruited to repress root identity in the legume symbiotic organ.

INTRODUCTION

Nodules are nitrogen-fixing organs that arise from root cortical cells in response to an external signal from free-living rhizobacteria. The capacity to form these symbiotic organs confers an ecological and agronomic advantage, which is restricted to a few plant lineages and represents a recent acquisition (40 to 60 million years ago) in the plant kingdom (Soltis et al., 1995). As this organ is formed facultatively during symbiosis, it also represents a fascinating model to study plant organogenesis. For these reasons, the legume root nodules have received a great deal of attention (Ferguson et al., 2010; Desbrosses and Stougaard, 2011; Oldroyd et al., 2011). Determining the mechanisms that govern the establishment and maintenance of this symbiotic organ is thus important for our understanding of the acquisition and construction of new organs.

The early molecular events of nodule initiation are now well understood (Oldroyd et al., 2011; Popp and Ott, 2011); however, less is known about the mechanisms that regulate nodule meristem identity and maintenance. Both *Medicago truncatula* and pea (*Pisum sativum*) have indeterminate nodules with an apical meristem that persists in nodules for a few weeks and continues to carry out cell division. Although both nodules and roots have an apical meristem, nodules are considered different from lateral roots because of their different cellular origin and vascular anatomy. However, several studies have noted shared features in the developmental programs of these two organs (Hirsch and Larue, 1997; Mathesius et al., 2000; de Billy et al., 2001), suggesting that legumes may have co-opted components of root organogenesis to construct a novel organ, the symbiotic nodule.

Homeotic mutants represent useful tools to understand the genetic regulatory pathways underlying organ development, identity, and maintenance. In pea, several floral and leaf homeotic genes have been characterized (Domoney et al., 2006), but *COCHLEATA* (*COCH*) remains unidentified. Among the pea mutants, *coch* represents an interesting case because several organ identities, including nodule identity (Voroshilova et al., 2003; Ferguson and Reid, 2005), are modified by the *coch* mutation (Marx, 1987; Yaxley et al., 2001). Here, we report the isolation and molecular characterization of the *NODULE ROOT* (*NOOT*) and *COCH* genes from *M. truncatula* and pea, respectively. The *noot* and *coch* mutants produce many abnormal nodules that develop roots from the apical part of the nodule. This suggests that the *NOOT* and *COCH* genes play an important role in the maintenance of the nodule developmental program. We show that *NOOT* and *COCH* are

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orthologs of the *Arabidopsis thaliana BLADE-ON-PETIOLE* (*BOP*) genes and hypothesize that *NOOT* and *COCH* were recruited from other developmental programs during legume evolution to participate in the coordination of the symbiotic nodule developmental program.

RESULTS

The *M. truncatula noot* Mutant Is a Nodule Homeotic Mutant

We identified four lines (tnk507, NF2717, NF4445, and NF5894) characterized by altered nodule development during screens of *M. truncatula Tnt1* transposon mutant collections (d'Erfurth et al., 2003; Tadege et al., 2008). In these mutant lines, the nodule can develop one or multiple roots in an apical position on the fully developed nodule (Figures 1A and 1B); thus, the *noot* mutants can switch from a nodule to a root developmental program. The molecular characterization of these insertion lines (see below) showed that they represent four alleles of the same gene. The emergence of roots from a small fraction of nodules can be detected macroscopically 2 to 3 weeks after rhizobium infection, and by 4 weeks, ~20% of nodules have developed roots. Careful observation of the mutant root system showed that several types of organs were observed. Mutant nodules can resemble wild-type nodules (Figure 1A) or have a root developing in an apical position (Figure 1B). Other mutant nodules are multilobed and can develop roots (Figure 1C). Histological studies of these mutant nodules showed an extended meristem region characterized by the proliferation of small apical cells (Figures 1D and 1E). Thus, these complex, multilobed nodules resulted from uncontrolled proliferation of the meristematic region in the mutant organs. The proportion of the different nodule types observed in *noot* varied from experiment to experiment and the form with roots increased with the age of the plant (see Supplemental Figure 1 online), but we were unable to determine the environmental factors influencing this variation.

The abnormal nodules observed on the roots of the *noot* mutant have the pink color characteristic of Leghemoglobin in functional nitrogen-fixing plants (Figure 1A). Consistent with this, we measured plant dry weight, commonly used as a proxy for measuring effectiveness of symbiosis (Terpolilli et al., 2008), and we showed that growth of mutant plants under symbiotic conditions is not adversely affected compared with wild-type plants (see Supplemental Table 1 online). In addition, histological observation of *noot* nodules (see Supplemental Figure 2 and Supplemental Table 2 online) showed that they have the characteristic zonation of wild-type nodules, are properly invaded by symbiotic bacteria, and contain differentiated symbiotic cells.

We also noticed that some nodules of the *noot* mutant were converted to root-like structures early in their development before the onset of nitrogen fixation. This early conversion resulted in organs that resemble roots, but with a swollen base that was infected by bacteria (Figure 1F), suggesting that conversion took place shortly after the nodule primordium stage. The presence of the rhizobia at the base of the hybrid structure was confirmed using green fluorescent protein (GFP)-labeled bacteria (Figure 1G). Some roots formed on mutant organs contain two vascular strands (Figures 1F and 1H).

Interestingly, root hairs were formed in the early converted structures described above (Figure 1I; see Supplemental Figures 3A and 3D online). This cell type is typically absent from wild-type nodules (Figure 1J) but could be observed on the mutant nodule-like structures in the vicinity of the vascular tissue (Figure 1K). Nodules can develop on the root emerging from abnormal nodules; furthermore, infection can be detected on the root hairs present in front of a symbiotic infected zone, demonstrating that these hybrid structures have cells that have the identity of a genuine root hair (see Supplemental Figure 3A online) and are competent for infection by bacteria (see Supplemental Figures 3B and 3C online).

To test if the *noot* mutant had altered hormone sensitivity, we compared mutant and wild-type root growth and root sensitivity to different hormones or medium composition. These experiments showed that *noot* was not affected in lateral root formation, ruling out the possibility that the mutant phenotype resulted simply from additional lateral root formation close (or adnate) to the symbiotic organ (see Supplemental Figure 4 online) nor was the *noot* mutant affected in root growth and root sensitivity to different hormones. In summary, these results show that the absence of *NOOT* allows nodule to root conversion at various times during nodule growth and development.

The Roots on the Mutant Organ May Originate from the Vasculature of the Nodule

In abnormal *noot* nodules, a root is generally present in an apical position, but some nodules have multiple roots emerging from the meristematic region (see Supplemental Figures 3D and 3E online). Staining mutant nodules with methylene blue (Figure 1F) shows that the mutant root vasculature connects with the nodule vasculature. Some roots formed on mutant organs contain two vascular strands (Figure 1H) connected to two nodule vascular strands (Figure 1F; see Supplemental Figure 3D online). We also observed nodules with two to four mutant roots in apical positions whose vasculature is continuous with the multiple vascular strands present in the apex of unilobed nodules (see Supplemental Figures 3D and 3E online). This led us to hypothesize that the roots present on *noot* nodules originate from the nodule vasculature rather than from the nodule apical meristem.

We explored this idea using an ProEnod11:β-glucuronidase (GUS) reporter construct (Boisson-Dernier et al., 2001) that is expressed in the nodule apical region as well as in the root tip in wild-type *M. truncatula* (Figures 1L and 1M; Journet et al., 2001; Svistoonoff et al., 2010). In *noot* plants, the expression of this fusion in the root tip is conserved (Figure 1M). In *noot* abnormal nodules, *GUS* expression in the nodule apical region is reduced or even absent, but patches of GUS staining can be seen, which are associated with the nodule vascular strands (Figures 1N and 1O). In early converted nodules, expression was observed in the vascular tissue connected to the emerging root tip (Figure 1O). Consistent with the methylene blue staining above, this indicates that the nodule root vasculature is continuous with the nodule vasculature. This experiment also shows that hybrid organs can have vascular strands with nodule identity (no *GUS* staining and not connected to a root) as well as vascular strands with changed identity that express the *GUS* marker, both at the same time.

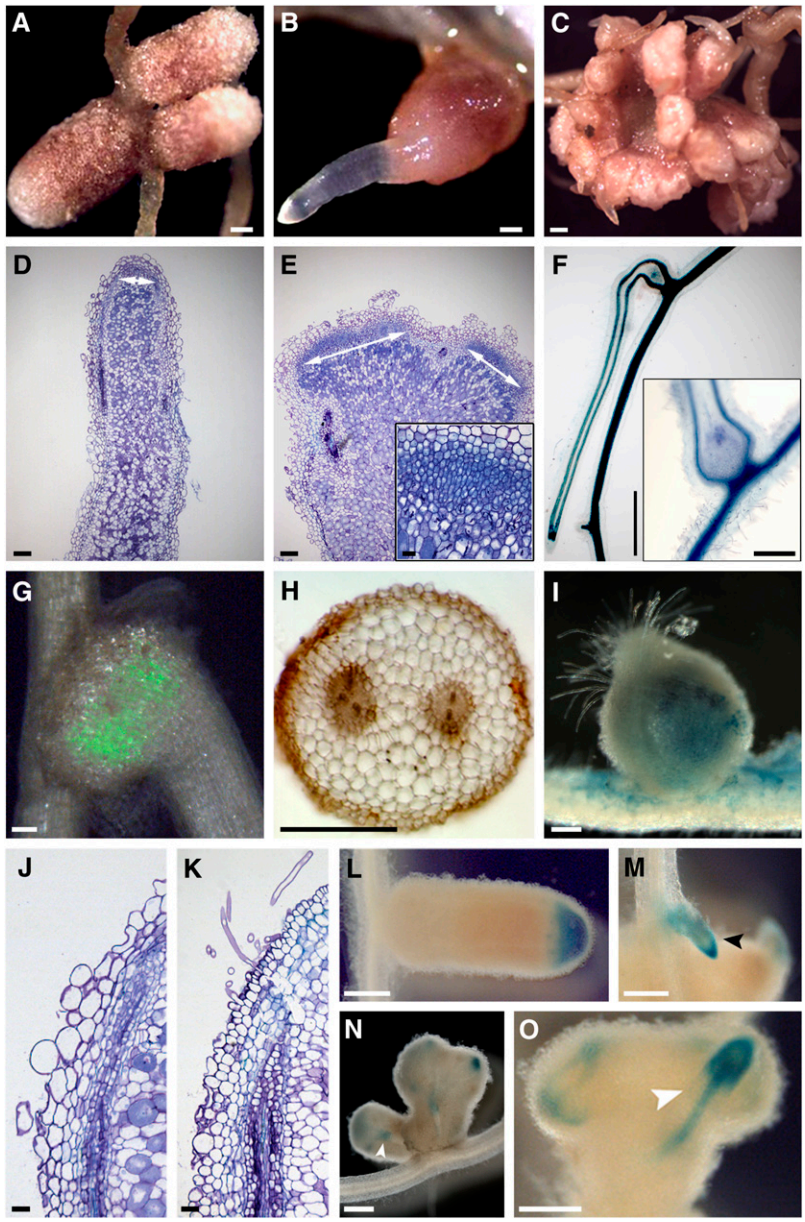

Figure 1. *NOOT* Is Necessary for the Maintenance of the Nodule Developmental Program.

(A) and **(B)** Nodules in wild-type **(A)** and *noot* **(B)** plants formed 15 d after inoculation. A root develops in an apical position on the *noot* nodule.
(C) A complex 6-week-old *noot* nodule. The complex mutant nodule exhibits enlarged nodule lobes harboring roots and callus-like tissue and can be observed in all mutant backgrounds.
(D) and **(E)** A longitudinal section through wild-type **(D)** and *noot* **(E)** nodules, showing enlargement of cell division territories (white bar) and perturbation of cell division polarity in the mutant meristematic region, despite the correct gradient of differentiation in the central tissue. The inset represents an enlargement of the meristematic region.
(F) Methylene blue staining of an early converted nodule showing the root part with double vascular tissue. The inset shows a close-up of the base of the nodule with the opaque region at the base of the mutant structure containing bacteria.
(G) Lateral root-like structure from *noot* inoculated with *S. meliloti* constitutively expressing GFP (green).
(H) Transverse section through a mutant nodule root, showing the presence of two vascular steles.
(I) to **(K)** Changes in cell identity in *noot* nodules.
(I) A young *noot* nodule with root-like tissues, including root hairs in the apical position. Blue staining indicates the presence of *LacZ* expressing bacteria.
(J) and **(K)** In the apical region facing the vasculature of the *noot* nodules, root epidermis (small squared) and root hair–like cells **(K)** replace the round-shaped cortical cells present in wild-type nodules **(J)**.

The *NOOT* and *COCH* Genes Are Orthologs

The *noot* phenotype is similar to the phenotype described for the pea *coch* mutant lines JI 2165 and JI 2757 (Ferguson and Reid, 2005), indicating that the pea and *M. truncatula* mutants could carry defects in the same gene. A large number of *coch* mutants have been described in pea (Table 1), some of which were shown to belong to the same allelic complementation group (Gottschalk, 1971; Rozov, 1992). We crossed lines *SGRcoch* and JI 1824 ($n = 2$) and JI 2757 and JI 1824 ($n = 10$) to confirm their allelism. The *coch* phenotype (presence of mutant nodules, stipules, and flowers) of the F1 plants demonstrated that they are allelic. The eight *coch* lines used in our study, including lines JI 2165 and JI 2757 (Yaxley et al., 2001; Ferguson and Reid, 2005), all develop mutant (*coch*) nodules. We observed early nodule-to-root conversions (see Supplemental Figure 3E online) and the presence of root hair cell types on *coch* mutant nodules. These phenotypes suggest that the mutation affects nodule development in the same way in both species.

The genetic analysis of the *noot* mutation in line tnk507 demonstrated that the mutation is monogenic recessive (see Supplemental Table 3 online). To facilitate the isolation of the *NOOT* gene, we hypothesized that the *M. truncatula NOOT* and pea *COCH* genes were orthologs and used the genetic map location of the *coch* mutation (Zhukov et al., 2007) to select a tagged locus in line tnk507. The *COCH* gene maps on pea chromosome 5 at a position syntenic to 57 centimorgans on *M. truncatula* chromosome 7. One flanking sequence tag (FST) from line tnk507 is positioned on BAC clone AC147961 in this region and corresponds to gene AC147961_14.2. This mutant locus is physically linked to the mutation (see Supplemental Table 3 online). Using a PCR approach, we confirmed that the same gene is *Tnt1* tagged in all four *noot* lines. *Tnt1* is inserted 175, 404, 2104, and 2338 bp after the *NOOT* AUG codon in lines tnk507, NF4445, NF5894, and NF2717, respectively (Figure 2A). The disruption of the same gene in these four independent *noot* lines demonstrates that it corresponds to the *NOOT* gene. The *NOOT* gene is 2810 bp long, composed of two exons and one intron (Figure 2A), and is predicted to encode a protein of 482 amino acids.

Using PCR, inverse PCR, and transposon display PCR techniques (Ratet et al., 2010), in combination with gene-specific and degenerate oligonucleotides, we amplified and sequenced the pea gene corresponding to *NOOT* in the SGE wild-type background. Using this information, we sequenced the gene from the wild-type progenitor lines of the eight *coch* mutants (Table 1). The putative *COCH* gene sequence is 100% identical at the nucleotide level in the different parental wild-type lines, suggesting a high level of conservation in these pea backgrounds. The encoded protein (483 amino acids) is 93% identical and 96% similar to NOOT. PCR analysis and sequencing the gene from the eight mutant lines showed that they correspond to three point mutants and five deletion mutants (Figure 2A, Table 1). The putative *COCH* gene could not be amplified in the pea deletion mutants (see Supplemental Figure 5 online) except for line JI 1824, which has a 2-bp deletion in the second exon (Figure 2A). This deletion introduces a frame shift at position 209 of the nucleotide sequence, resulting in a stop codon at position 266. Lines JI 2165 and *SGEapm* have mutations that introduce a stop codon at amino acid position 51 and 69, respectively. Mutant line Wt11304, described as a weak *coch* allele (*heterophyllus*; Swiecicki, 1989), has a C-to-T transition, resulting in a Ser-to-Phe change at amino acid position 387. The characterization of these mutations in the eight *coch* lines demonstrates that we have cloned the *COCH* gene. This analysis also confirmed our hypothesis that *NOOT* and *COCH* are orthologs.

The *NOOT*, *COCH*, and *BOP* Genes Define a Clade for the BTB/POZ-Ankyrin Domain Proteins

The *NOOT* gene encodes a BTB/POZ-ankyrin repeat protein of the NPR1 family (Figure 2A). Phylogenetic analysis (Figure 2B; see Supplemental Data Set 1 online) showed that the closest *Arabidopsis* homologs to *NOOT* and *COCH* are the *NPR*-like *BLADE-ON-PETIOLE1/BLADE-ON-PETIOLE2* (*BOP1/BOP2*) genes (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). NOOT shares 77% identity and 85.6% similarity with BOP2. NPR1 participates in salicylic acid–mediated plant defense against necrotrophic pathogens (Pieterse and Van Loon, 2004; Fu et al., 2012). By contrast, the *Arabidopsis bop1 bop2* double mutant develops blade-on-petiole structures and is altered in flower formation (Hepworth et al., 2005; Norberg et al., 2005). This double mutant is not known to have altered response to pathogens, which suggests that *NPR1* and *BOP* genes have distinct biological functions. Our phylogenetic analysis of NPR-like (BTB/POZ-ankyrin) sequences from different legume and nonlegume plants shows that two clades of BTB/POZ-ankyrin proteins exist in plants (Figure 2B). One clade includes the known At-NPR proteins (At-NPR1 to At-NPR4) and three putative NPR1-related proteins from *M. truncatula* (encoded by Mt2g046480, Mt2g034520, and Mt5g097890). This suggests that these three *NPR1*-related genes from *M. truncatula* may have functions in plant–microbe interactions. The other clade includes At-BOP1 and At-BOP2, NOOT, COCH, and their putative orthologs (Lj-NBCL, Gm-NBCLa, and Gm-NBCLb) from *Lotus japonicus* and soybean (*Glycine max*). A rice (*Oryza sativa*) protein (ABE11621.1) is also included in this clade, suggesting

Figure 1. (continued).

(L) Wild-type nodule expressing the *pEnod11*:*GUS* construct in apical position.
(M) Mutant *noot* root tip expressing the *pEnod11*:*GUS* construct (arrowhead). The same pattern is observed in wild-type root tips.
(N) and **(O)** Expression of the *pEnod11*:*GUS* fusion in the *noot* nodule apex and in vascular bundles (arrowheads). Note the expression of the fusion in the root tip region of the nodule root along the vasculature.
Bars =250 μm in **(A)** and **(B)**, 500 μm in **(C)**, 250 μm in **(D)** and **(E)** (inset in **[E]** = 50 μm), 3 mm in **(F)** (inset = 1 mm), 300 μm in **(G)** to **(I)**, 20 μm in **(J)** and **(K)**, and 600 μm in **(L)** to **(O)**.

Table 1. Characteristics of the *coch* Mutant Lines Used in This Study

Accession ID	Background	Mutagen	Type of Mutation	References
JI2165	Weitor	EI	AGA:TGA>Arg:$STOP_{51}$	Blixt (1967)
Wt11304	Paloma	EMS	TCT:TTT>Ser:Phe_{387}	Swiecicki (1989)
SGEapm	SGE	EMS	CGA:TGA>Arg:$STOP_{69}$	Zhukov et al. (2007)
JI1824	Dominant	FNB	Δ2 bp > frame $shift_{209}$ $STOP_{266}$	Wellensiek (1963)
SGRcoch	SGE	γ-Ray	Full-length deletion	Rozov (1992)
FN3185/1325	JI2822	FNB	Full-length deletion	This study
JI2757	Parvus	x-ray	Full-length deletion	Blixt (1967)
JI3121	DGV	x-ray	Full-length deletion	Gottschalk (1964)

EI, Ethylene Imine; EMS, ethyl methanesulfonate; FNB, Fast Neutron Bombardment.

the existence of BOP orthologs in monocots. The definition of this clade is supported by microsyntenic relationships between the *Arabidopsis*, soybean, and *M. truncatula* genomes, as indicated in Supplemental Figure 6 online. In addition, synteny is detected between *M. truncatula* and soybean, as well as between both of these legumes and *Arabidopsis*. Altogether, this analysis indicates that the divergence between the two clades predates the *Arabidopsis* and legume divergence but may also predate the divergence between monocots and dicots. We named this clade the NBCL (for NOOT/BOP/COCH like) clade of the BTB/POZ-ankyrin proteins.

The *BOP* genes from *Arabidopsis* encode nuclear-targeted proteins that act as complexes with transcription factors (Hepworth et al., 2005). To test whether *NOOT* is a functional ortholog of *BOP1* and *2*, we overexpressed *NOOT*, or a *NOOT*:*GFP* translational fusion, in *Arabidopsis*. The two constructs phenocopy *BOP* overexpression in *Arabidopsis* (see Supplemental Figures 7A to 7D online), as previously observed by Ha et al. (2007) and Norberg et al. (2005), demonstrating that *NOOT* and *BOP* are functional orthologs. In addition, the NOOT:GFP fusion is targeted to the nucleus, indicating that the NOOT protein is a nuclear protein (see Supplemental Figures 7E and 7F online).

The *NBCL* Clade Shares Conserved Developmental Functions

The pea *coch* alleles described in the literature were first isolated based on their stipule and flower phenotypes (Wellensieck, 1959). As various *coch* alleles are in different genetic backgrounds and were tested in different conditions, we reassessed the mutant pea lines described in Table 1 for their phenotypes under identical controlled growth conditions. Our analysis showed that all *coch* mutant lines have reduced or absent stipules in their first nodes (Figures 3A to 3D). The *coch* stipule phenotypes of mature plants vary from weak modifications (asymmetric shape; see Supplemental Figure 8 online) in mutant line Wt11304 to spoon-like leaf structures (lines JI 3121, *SGEapm*, and FN 3185; Figure 3F) and even complete conversion into complex pea leaf structures in lines JI 2165, JI 2757, and JI 1824 (see Supplemental Figure 8 online for an example of complete conversion). Apart from the weak Wt11304 allele that corresponds to an amino acid (Ser>Phe) modification, the penetrance of the stipule phenotype does not relate to the type of mutation (deletion of the complete genomic region versus truncated proteins) found in these mutants.

In addition to stipule modification, the *coch* mutation alters various aspects of flower development in all of these lines, as described previously by Yaxley et al. (2001). A wild-type pea flower is composed of a keel, two wings, and a standard (Figures 3G and 3I). The *coch* mutants we analyzed showed dorsalized flowers with two standards and chimeric stamen-wing petals (Figures 3H and 3J). In addition, bracts are more complex in mutant flowers (Figures 3K and 3L), and two pods resulting from the fertilization of two pistils were observed on some flowers (Figure 3M).

In *Arabidopsis*, the effect of the *bop* mutation (*bop1* dominant negative mutation or *bop1 bop2*) was first described as outgrowth of the leaf blade on petioles (Ha et al., 2003, 2004; Hepworth et al., 2005; Norberg et al., 2005). Flower symmetry is also affected, with the formation of additional organs (Ha et al., 2003; Norberg et al., 2005; Xu et al., 2010). Thus, the phenotypes of the pea and *Arabidopsis* mutants are similar, affecting the development of the proximal region of the leaf as well as the symmetry of the flower. This suggests a conservation of *NBCL* gene function in the development of these organs.

When we investigated the phenotype of leaves and flowers in the *M. truncatula noot* mutant, we found that stipules were simplified (Figures 4A to 4E). In wild-type plants, the number of serrations of the stipule increased from one to two digitations at the first node to three to four digitations after the third node (Figures 4C and 4D). Older plants can have a more complex serration of their stipules (Figures 4A and 4C). In all four *noot* alleles, the number of digitations remains low throughout the development of the plant (Figure 4E). Flower modification is subtle in *noot*, suggesting a reduced penetrance of the mutation in *M. truncatula*. Additional organs (petals and stamens) can be observed in flowers of the four *noot* alleles (Figures 4F to 4I).

The modified development of the flower and the leaf proximal region in pea, *M. truncatula*, and *Arabidopsis* indicates that the *NBCL* genes have a conserved function in aerial organs in eudicot plants.

The *NOOT* and *COCH* Genes Are Expressed during Symbiotic Organ Development

The *noot* and *coch* mutant phenotypes suggest that *NOOT* and *COCH* not only participate in the definition of the stipule identity and flower organ patterning but also play a role in the robust maintenance of nodule identity. The analysis of *NOOT* gene

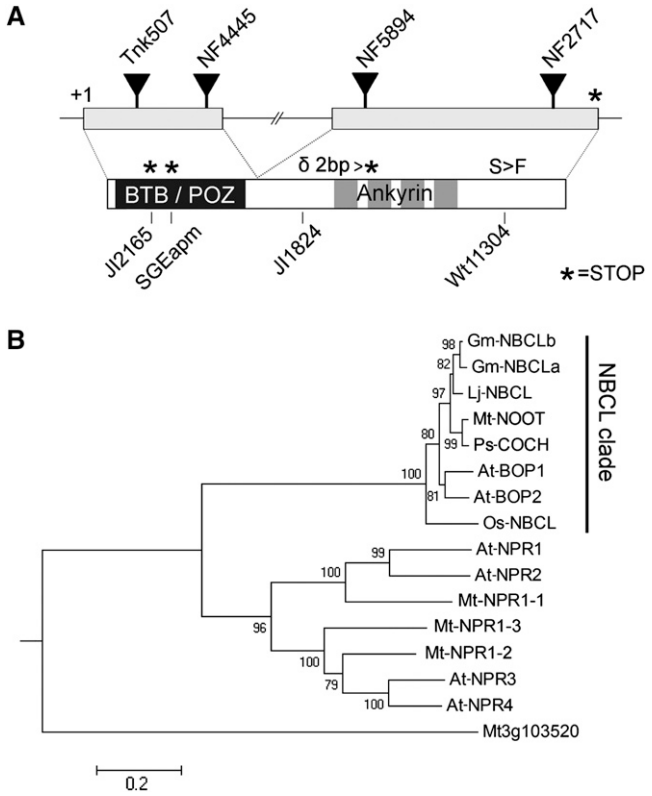

Figure 2. *NOOT* and *COCH* Encode a Bric-a-Brac Tramtrack and Broad complex/POx virus and Zinc finger (BTB/POZ) Ankyrin Protein.

(A) *NBCL* gene structure (top) and conserved domains of the corresponding protein (bottom). Exons are represented by light-gray rectangles. The two domains (BTB/POZ and Ankyrin) are indicated on the protein representation. The positions of the *M. truncatula noot* mutations (*Tnt1* insertions) are indicated by triangles. The positions of the pea *coch* mutations are indicated below the protein scheme. For each *coch* line, the consequence of the mutation is schematized over the protein representation.

(B) Phylogeny of the NBCL clade. The tree was built from aligned protein sequences using the neighbor-joining method, with branch support from 10,000 bootstrap replicates shown. Note the common ancestry of NOOT, BOP, and COCH among the NPR and NPR-LIKE family. At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Gm, *Glycine max*; Lj, *Lotus japonicus*, Ps, *Pisum sativum*; Mt, *Medicago truncatula*. The ankyrin repeat–containing protein Medtr3g103520 (http://phytozome.net/) was used to root the tree.

expression in different organs by quantitative RT-PCR (qRT-PCR) experiments agreed with the gene atlas data (http://mtgea.noble.org/v2/; Benedito et al., 2008) and showed that *NOOT* is expressed in all organs of the plant (Figure 5A). To further define expression during leaf/stipule development, we performed an in situ hybridization on the pea shoot apical meristem (SAM). This experiment (Figures 5B and 5C) shows that *COCH* gene expressed at the base of the developing leaf where stipules are formed, suggesting that COCH action is stipule primordium specific. *NOOT* expression in roots is variable between experiments (Figure 5A) but is induced in nodules compared with roots 10 d after infection. The qRT-PCR experiment shown in Figure 5C indicates that *NOOT* expression increased during the early stages of nodule development (from day 0 to 4) and remained stable later (day 6 to 10), when nodules were fixing nitrogen efficiently. Using a *ProNOOT*:*GUS* fusion expressed in transgenic plants, we detected the expression of the construct in the upper part of the nodule in cell layers surrounding the distal part of the vascular tissues (Figures 5D to 5F). In addition, in the transgenic *M. truncatula* plants, the *ProNOOT*:*GUS* fusion is expressed in the root central stele, similarly to *Arabidopsis BOP1* and *BOP2* (see Supplemental Figure 9 online). These patterns of expression are compatible with an organ autonomous action of *NOOT* and *COCH* and with the different phenotypes of the mutants. To further prove that the *NOOT* action is local, we grafted *noot* roots onto wild-type scions. These hybrid plants retained the *noot* phenotype, demonstrating that the *NOOT* in the aerial part of the plant cannot rescue the phenotype. Taken together, our work indicates that the action of *NOOT* in nodules does not involve long-distance signaling.

DISCUSSION

In this study, we identified *NOOT* and *COCH* and described the corresponding mutants. The *noot* mutant was isolated from *Tnt1*-tagged *M. truncatula* mutant collections (d'Erfurth et al., 2003; Tadege et al., 2009) as lines with nodules developing a root in the apical position. The *coch* mutants were first described as stipule and flower developmental mutants isolated from a neutron-irradiated population (Wellensieck, 1959); only later was it found that the mature nodules of these mutants develop roots in the apical position (Voroshilova et al., 2003; Ferguson and Reid, 2005). Making use of the *Tnt1*-tagged *M. truncatula* mutants, we characterized *NOOT* and showed that it is orthologous to *COCH*. Our work has thus identified *COCH*, which was used as a classical genetic marker in pea and studied for its role in pea leaf development (Weeden et al., 1998; Gourlay et al., 2000).

NOOT and *COCH* are orthologous to *Arabidopsis BOP1* and *BOP2* and belong to the large NPR1-like BTB/POZ-Ankyrin domain protein family. Our phylogenetic analysis demonstrates that this family can be divided into two clades: One includes the NPR1-like proteins that probably have a role in the plant–pathogen interactions; the other includes *NOOT*, *BOP*, and *COCH*. We have named this clade the *NBCL* family.

Arabidopsis BOP1 and *BOP2* have been shown to be important regulators of growth and development of lateral organs and are expressed at the leaf/meristem boundary (Norberg et al., 2005; Khan et al., 2012). They also participate in the regulation of ad/abaxial polarity, are required for normal gene expression at the meristem/primordium boundary, affect phyllotaxy, and regulate meristem activity (reviewed in Barton, 2010); however, their molecular function remains unclear. They may work as transcription factors (Jun et al., 2010), form part of transcription factor complexes (Hepworth et al., 2005; Rochon et al., 2006), and/or interact with the proteasome through the BTB/POZ domain as a member of the E3 ligase complex for Cullin3-based protein degradation (Pintard et al., 2004; Gingerich et al., 2007).

The *coch* mutation affects both stipule and flower development in pea (Gourlay et al., 2000; Yaxley et al., 2001). The mutation suppresses stipule formation at the first few nodes and changes

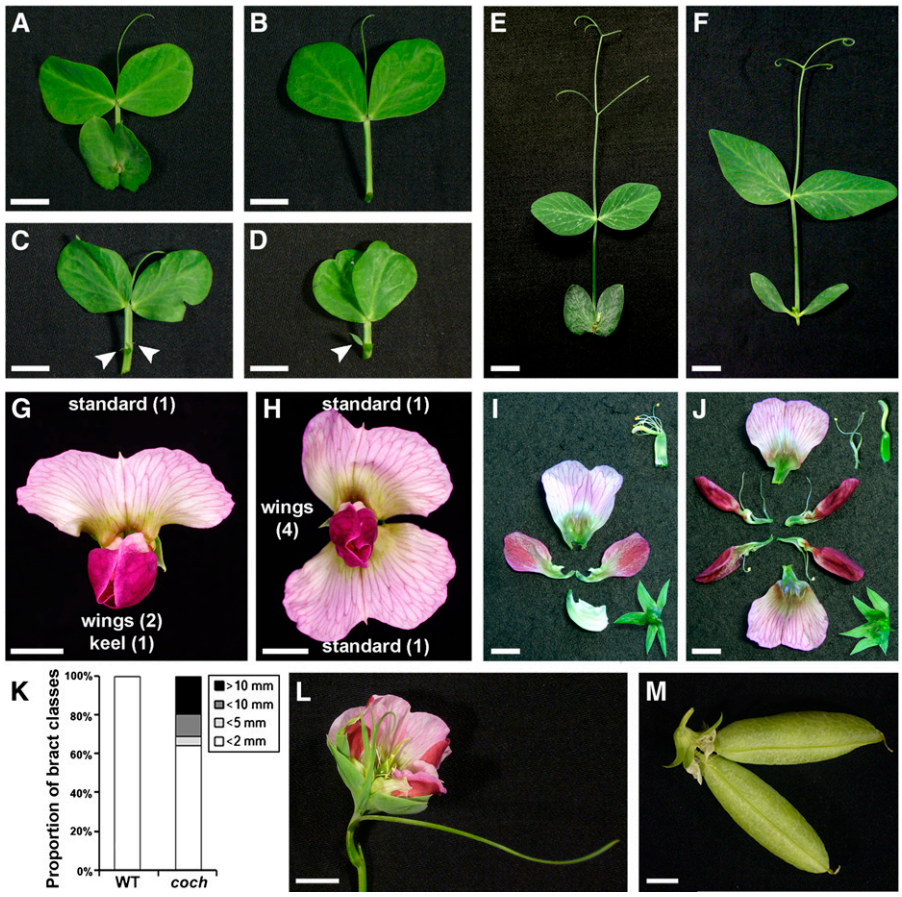

Figure 3. Aerial Organ Modification in *coch*.

(A) to **(J)** Wild-type (SGE; **[A]** and **[E]**) and *coch* (*SGEapm*; **[B]** to **[D]** and **[F]**) pea stipules from the third (**[A]** to **[D]**) and sixth (**[E]** and **[F]**) nodes. Leaves from the first to fifth nodes bear no stipule or extremely reduced structures on nodes 3, 4, and 5 (arrowheads in **[C]** and **[D]**). Leaf-like structures appear later in development **(F)**, and stipules are further converted into compound blades with leaflets and tendrils. All *coch* lines investigated exhibit these modifications, except for the Wt11304 *coch* line, which exhibits asymmetrical stipules and has also been described as the *heterophyllus* mutant (Rozov, 1992). The colored corolla of a pea wild-type (SGE) flower (**[G]** and **[I]**) is composed of a fused pair of keel petals enclosed between two dark-colored wings (bottom part of **[I]**) and a standard. A typical adaxialized *coch* (*SGEapm*) flower (**[H]** and **[J]**) with a double standard and four chimeric stamen-wing colored petals **(J)**. Floral modifications are constant among all *coch* alleles tested except for Wt11304, a weak *coch* allele with less pronounced flower symmetry modifications.

(K) Length of the subtending bract in *coch* and wild-type (WT) plants. Bracts were measured on mature pods from *P. sativum* and separated into four different classes (0 to 2, 2 to 5, 5 to 10, and >10 mm). Each class was represented as the percentage of total bract (n = 77 and 67 for the wild type and *coch*, respectively).

(L) Example of bract (arrowhead) conversion in *coch*.

(M) Double pods from a single *coch* flower.

Bars = 1 cm in (A) to (J), (L), and (M).

organ identity at the later nodes, into leaflet-like organs or more complex structures bearing leaflets and tendrils. The replacement of stipules with complex structures in *coch* can be interpreted as a reduced determinacy of this organ (Yaxley et al., 2001). Our work shows that the type of structure (leaflet-like or leaf-like) that replaces the stipule is not dependent on the mutant allele but is rather genotype dependent, suggesting interactions with other genes or proteins. Gourlay et al. (2000) showed that *COCH* represses blastozone identity in wild-type stipule primordia by inhibiting *UNIFOLIATA* expression. Thus, *COCH* helps to define the territory necessary for the correct acquisition of stipule identity in pea. In the *M. truncatula noot* mutant, the stipules have a simplified structure compared with the wild type, demonstrating a role for *NOOT* and *COCH* in the same organ, even if the mutation in *M. truncatula* does not change the identity of this organ but rather retains it in a juvenile state. We found that the *coch* mutation results in dorsalization of the flower in all *coch* lines tested. In addition to the dorsalized organs, supernumerary and fused organs are also present in the mutant flowers (Yaxley et al., 2001), including flowers with double pods, a character that resembles the double flowers reported for the corresponding *Arabidopsis bop* mutants (Xu et al., 2010). In *noot*, flower patterning is also

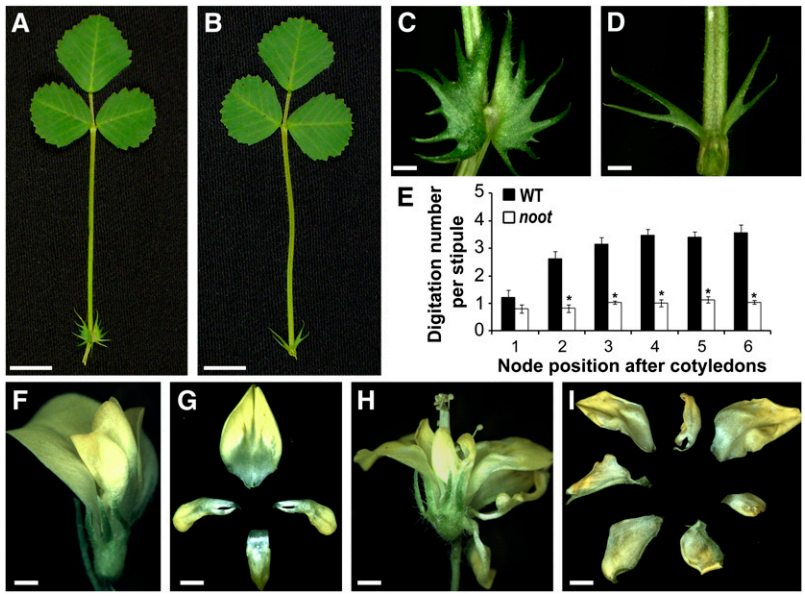

Figure 4. Aerial Organ Modification in *noot*.

(A) to **(D)** Wild-type stipules of *M. truncatula* are shown in **(A)** and **(C)**. Stipules are serrated with n serrations defining n+1 digitations. Mutant *noot* stipules are less serrated and reduced in area (**[B]** and **[D]**). Stipules are from the third node from the apex of 2-month-old plants.

(E) The number of digitations per stipule on the first six nodes of wild-type (WT; black bar) and *noot* mutant (white bar) plants. Data are presented as means with error bars representing the 5% confidence interval for each background (α = 0.05; n = 30). Asterisks indicate a significant difference between the mutant and the wild type using a nonparametric statistical test for independent sample comparisons (α = 0.05). Results are representative of three biological repeats obtained with two independent *noot* alleles (tnk507 and NF2717).

(F) and **(G)** Wild-type flower of *M. truncatula*.

(H) and **(I)** Floral modifications in *noot* were observed for three consecutive years in all *noot* lines grown under greenhouse conditions during the first 2 months of flowering (August to September 2009, 2010, and 2011, Gif-sur-Yvette, France).

Bars = 1 cm in **(A)** and **(B)** and 1 mm in **(C)**, **(D)**, and **(F)** to **(I)**.

altered. This proliferation of organs was also interpreted as reduced determinacy by Yaxley et al. (2001).

The molecular identification of *NOOT* and *COCH* genes as orthologs of the *BOP* genes brings together unnoticed similarities between the legume (*noot* and *coch*) and *Arabidopsis* (*bop*) mutant phenotypes, suggesting a conserved role of the *NBCL* genes in defining meristem territories in flower and leaf lateral organ development (Barton, 2010). It is interesting to note that the three NBCL genes are expressed in roots (see Supplemental Figure 9 online) but that none of these mutants (*bop*, *noot*, or *coch*) have root phenotypes under the tested conditions.

In legumes, the role of the *NBCL* genes in the development of the symbiotic organ is apparent. The most striking feature of *noot* and *coch* (Ferguson and Reid, 2005) nodules is the development of one to four roots in an apical position on the nodule. Our histological analysis suggests a vascular tissue origin of these roots and that the transition (homeosis) from nodule to root can take place at different stages of nodule development, indicating a role for the *noot*/*coch* genes immediately after primordium formation (i.e., at the time of establishing organ identity) and lasting throughout nodule development. In both the *M. truncatula* and pea mutants studied here, we observed a range of nodule-root phenotypes, including wild-type nodules (no homeosis), hybrid N_2-fixing nodules with a root in an apical position, complex nodule-like structures, and roots with swollen rhizobium-infected bases, corresponding to an early homeosis, not previously described in pea. The different nodule types, including wild-type-like nodules, indicate that the gene is not essential for nodule identity but rather participates in the definition and maintenance of this symbiotic organ.

Another characteristic of the *noot* mutant is the presence of nodules with multiple and enlarged meristematic regions. Histological examination showed that the orientation of cell division planes in the mutant nodule meristem is altered. This phenotype is reminiscent of the enlarged *clavata3* SAM mutant meristem in *Arabidopsis* (Clark et al., 1995). A similar phenotype was observed by Ferguson and Reid (2005) in three *coch* alleles and was confirmed for all of the pea lines tested in our study. This enlarged meristem is consistent with the presence of supernumerary organs in the flowers of *noot* and *coch* plants and points to a possible link between the *NOOT*/*COCH* and the *WUSCHEL*/*CLV3-ESR* related gene family (*WUS*/*CLE*) regulatory loop that controls stem cell activity. A relationship between *BOP* and stem cell activity was also suggested by Norberg et al. (2005).

We can hypothesize that the NBCL functions described above are also relevant in nodules, thus suggesting a parallel between the functioning of the SAM and the nodule meristematic region. Cytokinins are required in the SAM central zone for the maintenance of the stem cell pool. In *Arabidopsis*, cytokinins stimulate *WUS* and repress *CLV* genes in a regulatory loop

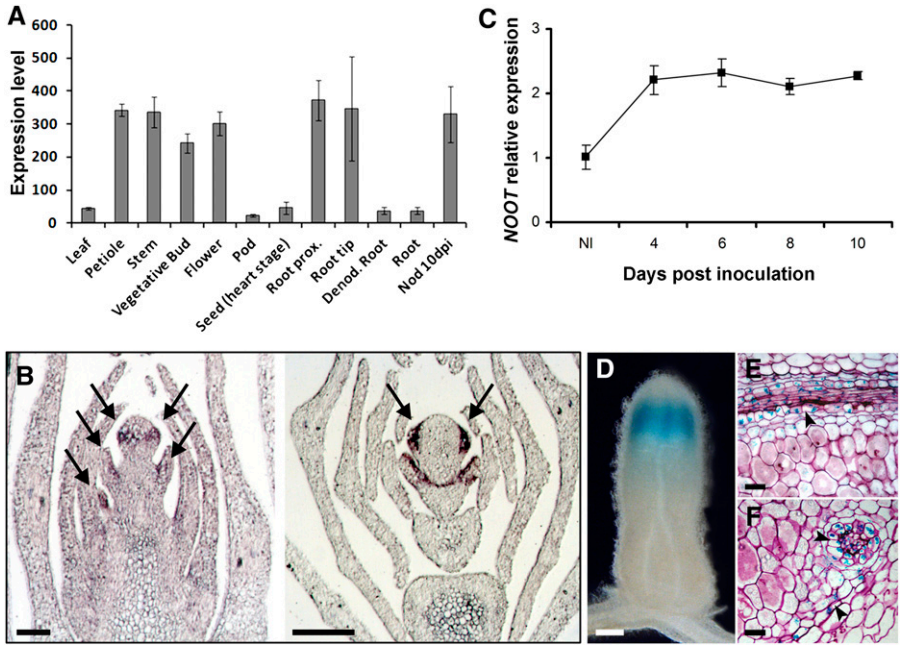

Figure 5. Expression of *NOOT* and *COCH* in Different Organs.

(A) *NOOT* is expressed in different organs. Values correspond to the level of Mtr.19586.1.S1_at Affymetrix probe signal from the Medicago Gene Expression Atlas (Benedito et al., 2008). Data and error bars represent means ± the confidence ($\alpha = 0.05$; $n = 3$). Root prox, 1 cm adjacent to the root tip; root tip, 5 mm; Denod root, denodulated roots at 28 d after inoculation (dpi); Root, 28-d-old root.

(B) *COCH* in situ hybridization in pea vegetative shoots seen in longitudinal (left panel) and transverse (right panel) sections. Arrows indicate the presence of the antisense signal in stipule primordia. The sense RNA used as a control gives no signals.

(C) *NOOT* expression during nodulation. NI (not inoculated) corresponds to day 0 of the experiment. Data and error bars represent means ± the confidence of three technical replicates representative of two biological replicates ($\alpha = 0.05$; $n = 3$).

(D) Expression of the *ProNOOT*:*GUS* construct in nodules of transgenic plants.

(E) and **(F)** Longitudinal **(E)** and transverse **(F)** sections showing expression in the nodule vasculature and endodermis boundary (arrowheads).

Bars = 200 μm in **(B)**, 250 μm in **(D)**, and 10 μm in **(E)** and **(F)**.

necessary for optimal meristem functioning (Barton, 2010). This regulatory network seems to be conserved in *M. truncatula* (Chen et al., 2009). In legumes, cytokinins are also necessary for the functioning of the nodule meristem, and cytokinin signaling genes are expressed preferentially in the meristematic region (Crespi and Frugier, 2008; Plet et al., 2011). Thus, with respect to this character, the SAM and nodule meristem are equivalent, in agreement with the hypothesis of Hirsch and Larue (1997) that suggests that nodules might have shoot characters. We can then speculate that *NOOT* and *COCH* genes participate in the definition of cellular territories in the nodule and more precisely in the communication between the central meristematic zone and the lateral regions defined by the cortex and the vascular tissues. If NOOT and COCH regulate (or interact with) stem cell activity in the meristem, as suggested by the meristem enlargement observed in the *noot* and *coch* mutant nodules, then we can hypothesize that a WUSCHEL-HOMEOBOX/CLE regulatory loop operates in the nodule meristem. In support of this hypothesis, Mortier et al. (2010) have shown that at least two *CLE* peptides are expressed in the meristematic region of the nodule. It will thus be interesting to know if the cytokinin-induced Mt-*WUSCHEL* described by Chen et al. (2009) is also expressed in the nodule meristem.

In addition, we propose that, through their role in domain definition, NOOT and COCH may participate in repressing the root identity of nodule vasculature initials. This could explain the development of the mutant roots in the region containing these vasculature initials and is consistent with the expression pattern of the *ProNOOT*:*GUS* construct in the region surrounding the vasculature initials. In agreement with this, Osipova et al. (2012) have shown that a ProWOX5:GUS transcriptional fusion, which is a marker for the root meristem, is expressed in vascular initials of mature nodules. Our work with the ProEnod11:GUS fusion also shows that the different vascular strands of a single nodule can have different identities (nodule or root). This independence suggests that the nodule vasculature is ontologically related to several roots rather than a single root.

Symbiotic organs from nonlegume plants (actinorhizal plants and *Parasponia*) are modified roots (Hirsch and Larue, 1997) and may represent intermediates or convergent forms in nodule evolution. Consistent with this hypothesis, it has been shown that both Nod and Myc symbiotic factors, in addition to their role in host recognition and nodule organ formation, stimulate lateral root development (Oláh et al., 2005; Maillet et al., 2011). This effect on lateral root development may represent their ancestral function, suggesting that nodule organogenesis programs have

evolved by co-option of lateral root developmental programs triggered by Nod factor–like signaling molecules. This hypothesis is supported by the phenotypic and molecular description of the *noot* and *coch* mutants and suggests that in legumes, the NOOT/COCH function used in eudicots to coordinate leaf and flower determinacy was recruited during evolution to coordinate nodule territories and, by doing so, control nodule symbiotic organ identity. In summary, our characterization of NOOT-mediated homeotic transformation has elucidated one element of the evolutionary origin of the nodule, showing a clear example of the recent co-option of elements from diverse regulatory pathways (tinkering) in the formation of a novel organ (Jacob, 1977).

METHODS

Plant Material

Medicago truncatula noot mutant lines tnk507, NF2717, and NF5984 were identified by a forward genetics screen of *Tnt1* insertion lines (Institut des Sciences du Végétal, France; Noble Foundation, Ardmore USA). NF4445 was obtained by reverse genetics screening using pools of genomic DNA from the Noble Foundation *Tnt1* mutant collection (Tadege et al., 2008). All these lines are derived from the *M. truncatula* R108-1 ecotype. JI 116, 932, 2822, 2413, 2165, 2758, 1824, 2459, 3121, and FN3185/1325 pea (*Pisum sativum*) lines are from the John Innes Centre germplasm (www.jic.ac.uk/GERMPLASM/). The genetic background and origin of these lines are summarized in Table 1. The *SGEapm* pea mutant is an ethyl methanesulfonate mutant isolated from a mutagenized population (A.Y. Borisov laboratory, All-Russia Research Institute for Agricultural Microbiology; Voroshilova et al., 2003). Wt11304 and *SGRcoch* mutant are described by Rozov (1992). *M. truncatula* nodulation experiments were performed on buffered nodulation medium (BNM; Ehrhardt et al., 1992) in growth chambers as previously described (Cosson et al., 2006). Characterization of the late nodulation phenotype, dry shoot weight, and hairy root experiments were done using a sand/perlite mix (1:3, v/v) in the greenhouse with a 16-h light period at 24°C and 60% humidity. Nodulated peas were grown in a sand/perlite mix (1:2 v/v) in growth chambers with a 16-h light period at 24°C and 60% humidity. Solution I was used for watering nodulating plants (EMBO manual; http://www.isv.cnrs-gif.fr/embo01/manuels/pdf/module1.pdf). Tnk507 designates the F3 generation resulting from the backcross of a tnk507 R2 plant with wild-type R108-1. Other *noot* alleles used in this study correspond to the R3 generation.

Bacterial Strains

M. truncatula and pea plants were inoculated with *Sinorhizobium meliloti* strains *Sm1021* (Galibert et al., 2001) and *Rlm3841* (Johnston and Beringer., 1975), respectively. *S. meliloti* strains were grown for 2 d at 30°C in tryptone yeast medium supplemented with $CaCl_2$ (10 mM final concentration) and appropriate antibiotics. The *Sm1021* strain harboring the *pDG71* (Gage, 2002) plasmid was used for constitutive *GFP* expression. *S. meliloti NifH*:*LacZ* used in this study was described by Szeto et al. (1984).

Analysis of Root Architecture

Plants were grown for 2 d on nitrogen-rich medium after germination. For 1-aminocyclopropane-1-carboxylic acid, Suc, indole-3-acetic acid, and 6-benzylaminopurine treatments, half of the seedlings were randomly transferred to treatment-free BNM media and the other half to BNM media containing the treatment. Lateral root analysis was done on nitrogen-rich media. Root length and the number of lateral roots were scored daily. Images were acquired before any root tip reached the base of the dish. The size of roots was recorded using ImageJ freeware (http://rsbweb.nih.gov/ij/).

DNA and RNA Extraction and DNA Amplification

Plant genomic DNA was extracted from leaves as described by d'Erfurth et al. (2003). Total RNA was extracted using the RNeasy Kit (Qiagen). First-strand cDNA was synthesized after DNaseI treatment followed by a reverse transcription reaction using Fermentas enzymes EN0521 and K1652. DNA amplification for genotyping was done using Eurobio Taq polymerase, and fragments for cloning and sequencing were amplified using TaKaRa ExTaq polymerase.

Gene Expression Analyses

Real-time RT-PCR analyses were performed as described by Plet et al. (2011). The reference genes used for *M. truncatula* qRT-PCR analysis were reported earlier by Gruber et al. (2009). The oligonucleotides used for amplification are listed in Supplemental Table 4 online. In silico gene expression analysis was performed using the websites http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Winter et al., 2007) and http://mtgea.noble.org/v2/.

Cloning and Sequencing of the *NBCL* Genes

Pea, *M. truncatula*, and soybean (*Glycine max*) sequences were retrieved using independent high-fidelity PCR products and then sequenced by GATC Biotech Company. Ps-*COCH* genomic sequences were identical in all investigated wild-type lines (JI 116, JI 932, JI 2413, JI 2822, and SGE). *NOOT* and *COCH* splice sites were determined by sequencing the corresponding cDNAs. *NOOT* gene FST in tnk507 was first isolated as described (Ratet et al., 2010) with the following modification: LTR6 and LTR5 oligonucleotides were used instead of LTR4 and LTR3, respectively. *Tnt1 NOOT* insertion sites for NF2717, NF4445, and NF5894 were isolated using combinations of *Tnt1* primers (LTR4 and LTR6) and compatible *NOOT*-specific primers (see Supplemental Table 4 online). *Tnt1* FST sequences are available at http://medicago-mutant.noble.org/mutant/. Sequencing of Gm-*NBCLb* for phylogenetic analysis was done using Gm19-RF and Gm19-FF primers (see Supplemental Table 4 online). Lj-*NBCL*, annotation BAC AP006393.1, was supported by EST.

Phylogenetic Analysis

The full-length protein sequences of the NBCL family were aligned using ClustalW (Thompson et al., 1994). The alignment was not corrected manually. The phylogenetic tree was built using MEGA4 program (Tamura et al., 2007). The evolutionary story was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The outgroup protein used for the analysis originated from *M. truncatula* and has a common ancestor to the NPR-like proteins as shown by the percentage of identity and the absence of synteny.

The *NBCL* syntenic relationship was constructed using the Plant Genome Duplication Database (Tang et al., 2008).

Plasmid Construction

To create a *Pro35S*:*NOOT* construct, a *NOOT* genomic fragment was amplified using NOOT-XhoI-3′ and NOOT-XhoI-5′ primers (see Supplemental Table 4 online) and then introduced in the *pGEMTeasy* vector. The resulting *Xho*I fragment was introduced into the *Xho*I unique site of a modified *pCAMBIA1390* vector containing the cauliflower mosaic virus *35S* promoter. For the C-terminal NOOT:GFP fusion, a genomic

fragment was amplified using NOOT-NcoI-3′ and NOOT-NcoI-5′primers (see Supplemental Table 4 online) and then introduced into the *Nco*I unique site of the *pCAMBIA1302* vector.

For the *ProNOOT*:*GUS* fusion construct, a 1.9-kb fragment upstream of the *NOOT* start codon was amplified using pNOOT-NcoI and pNOOT-EcoRI primers (see Supplemental Table 4 online) and then introduced into the *pGEMTeasy* vector according to the manufacturer's protocol. This fragment was used to replace the cauliflower mosaic virus *35S* promoter controlling *GUS* expression in the *pCAMBIA3301* vector, using *Nco*I and *Eco*RI unique restriction sites. The expression pattern of this construct was validated by complementing the *noot* mutation using the hairy root system (see below) with a genomic fragment containing the same promoter region plus the coding region of *NOOT*.

Plant Transformation

Arabidopsis thaliana (Columbia-0) transgenic plants were obtained following the floral dip method (Clough and Bent, 1998). Selected homozygous plants from the T3 and T2 generation were used for expression analysis and GFP localization, respectively. *M. truncatula ProNOOT*:*GUS* transgenic R108-1 plants were generated as described (Cosson et al., 2006) using the EHA105 *Agrobacterium tumefaciens* strain harboring *ProNOOT*:*GUS* in *pCAMBIA3301*. Fifteen different lines regenerated from independent calli were studied in the R1 generation. They all showed a similar pattern of GUS expression. Four independent lines were used later for imaging and sectioning. Hairy root transformations were done as described by Boisson-Dernier et al. (2001) using *Agrobacterium rhizogenes* strains Arqua1 containing the *pEnod11*:*GUS* plasmid. The transformed plantlets were transferred into sand/perlite medium for nodulation, as described above.

Light Microscopy and Sample Preparation

For GUS and LacZ reactions, nodules were incubated for 30 min in phosphate buffer (50 mM, pH 7.2) containing potassium ferricyanide and ferrocyanide (1 mM each) and X-Gluc or X-Gal (1.25 mM). The GUS reaction mix also contains SDS (0.1%) and EDTA (1 mM). Samples were incubated at 37°C for 2 to 6 h and then fixed under a vacuum for 2 h in 5% glutaraldehyde phosphate buffer (50 mM, pH 7.2). Samples were embedded in Technovit 7100 resin (Heraeus). Then, 7-µm sections were obtained using a Leica Microtome RM 2155 (Leica Microsystems) and counterstained for 5 min with 0.05% toluidine blue or ruthenium red for GUS or LacZ samples, respectively. Slides were then mounted in Eukit and observed on a Leica DMI6000B microscope equipped with a Leica DFC300 FX digital camera. For semithin sections, nodules were embedded in 4% agarose and sliced into 70-µm sections with Leica vibratome VT 1200S (Leica Microsystems). Whole-mount images of nodules were observed using a Leica M205FA fluorescent stereomicroscope (Leica Microsystems). Methylene blue staining of nodules was performed as described by Truchet et al. (1989). *COCH* in situ hybridization was performed using a probe of the CO5-COTAA PCR product (see Supplemental Table 4 online for primers) cloned into the *pGEMTeasy* vector as described (Gourlay et al., 2000). GFP localization was observed using a confocal microscope (Leica SP2 confocal microscope) with a band-pass filter (488-nm excitation line of a krypton/argon laser).

Flow Cytometry

For each genotype, three pools of 10 15-d-old nodules, arising from 10 independent plants, were prepared as described (Maunoury et al., 2010), except that filtered nuclei were stained with propidium iodide (50 µg/mL) and measured using a CyFlow SL3 flow cytometer. The endoreduplication index calculation was defined previously (Maunoury et al., 2010), and profiles were analyzed with Partec FloMax software.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers:genomic and cDNAs: *NOOT* (AC147961_14.2); *NOOT* cDNA (R108-1 ecotype, JN180858; A17 ecotype, JN180859); *Ps-COCH* gene and cDNA (JN180860 and JN180861); *coch* mutant genomic sequences (Wt11304, JN18062; JI 2165, JN18063; JI 1824, JN18064; and *SGEapm*, JN18065); *Gm-NBCLb* gene and cDNA (JN180866 and JN180867); Lj-*NBCL* (JN408495).Protein accession numbers are as follows: Mt-NOOT, ABD28327.1; At-BOP1, CBD23301.1; At-BOP2, AAC78536.1; Os-NBCL, ABE11621.1; Gm-NBCLa, Glyma03g28440.1; Gm-NBCLb, JN180867; Lj-NBCL, JN408495; Ps-COCH, JN180861; Mt-NPR1-1, AES65476.1; Mt-NPR1-2, AES64715.1; Mt-NPR1-3, AET00321.1; At-NPR1, NP176610.1; At-NPR2, NP_194342; At-NPR3, NP_199324; A-tNPR4, NP_193701.2; Medtr3g103520.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Nodule Number and *nodule root* Phenotype Penetrance.

Supplemental Figure 2. Organ and Symbiotic Cell Colonization Are Not Affected in *noot* Nodules.

Supplemental Figure 3. Identity Changes of the *noot* Nodules.

Supplemental Figure 4. Root Elongation, Root Inhibition by Hormones, and Lateral Root Formation Are Not Modified by the *noot* Mutation.

Supplemental Figure 5. Characterization of *COCH* Deletion Lines.

Supplemental Figure 6. Syntenic Relationship between *M. truncatula*, *Arabidopsis*, and *G. max NBCL* Loci.

Supplemental Figure 7. *NOOT* Overexpression Affects *Arabidopsis* Development and NOOT Localizes in the Nucleus.

Supplemental Figure 8. Stipule Phenotypes of the *coch* Mutants.

Supplemental Figure 9. Expression of the *NBCL* Genes in Roots.

Supplemental Table 1. Dry Shoot Weight of the Wild-Type and *noot* Lines Grown in Symbiotic Conditions.

Supplemental Table 2. Symbiotic Cells Are Differentiated in the *noot* Mutant.

Supplemental Table 3. Genetic Analysis of the *noot* Mutation.

Supplemental Table 4. Oligonucleotides Used in This Study.

Supplemental Data Set 1. Alignment Used to Generate the Phylogeny Presented in Figure 2B.

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AUTHOR CONTRIBUTIONS

A.Y.B., I.T., J.-M.C., K.S.M., S.M., P.R., and T.H.N.E. designed the research. G.A.H., J.H., J.-M.C., J.P., J.W., M.A., M.T., S.M., V.C., and V.Z. performed research. J.H., J.-M.C., J.P., M.A., M.T., P.R., T.H.N.E., and V.Z. analyzed data. J.H., J.-M.C., J.P., P.R., T.H.N.E., and V.Z. wrote the article.

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